for elucidating the disease mechanism and for providing more accurate disease diagnosis.

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Detection of 11 germline inactivating *TP53* mutations and absence of *TP63* and *HCHK2* mutations in 17 French families with Li-Fraumeni or Li-Fraumeni-like syndrome

EDITOR—The Li-Fraumeni syndrome (LFS) represents one of the most devastating genetic predispositions to cancers. This rare syndrome, affecting children and young adults, is characterised by a wide spectrum of early onset malignancies including bone and soft tissue sarcomas, brain tumours, adrenocortical tumours, and premenopausal breast cancers.[1] LFS was initially defined using stringent criteria[2]: (1) a proband with a sarcoma diagnosed before the age of 45, (2) a first degree relative with cancer before the age of 45, and (3) another first or second degree relative with either a sarcoma diagnosed at any age or any cancer diagnosed under the age of 45. Subsequently, Birch *et al*[3] defined Li-Fraumeni-like (LFL) syndrome as a proband with any childhood tumour or sarcoma, brain tumour, or adrenocortical tumour under 45 years, plus a first or second degree relative with a typical LFS tumour at any age and another first or second degree relative with any cancer under the age of 60. Eeles[4] proposed more relaxed criteria for LFL: a clustering of two typical LFS tumours in subjects who are first or second degree relatives at any age. Since the original reports of germline mutations of the tumour suppressor gene *TP53* in LFS,[5 6] numerous studies have shown that germline *TP53* mutations can be detected in approximately 70% of LFS families and 20% of LFL families,[1] suggesting the possible involvement of other genes in LFS. This hypothesis was recently confirmed by the detection, in one LFS family and one family suggestive of LFS, of germline mutations of *hCHK2*, the human homologue of the *Saccharomyces cerevisiae RAD53* gene, located on chromosome 22q12.[7 8] *hCHK2* encodes a kinase, which is able to phosphorylate, in response to DNA

damage, the Cdc25C phosphatase involved in the G2 checkpoint[7] and TP53.[9–11] We considered that the *TP53* homologue, *TP63*/*p51*/*KET*[12–14] located on 3q27-29, was also a candidate gene for the LFS not associated with the germline *TP53* mutation, since the isotype p63γ transactivates reporter constructs containing the *TP53* binding sites present within *p21*/*WAF1*/*CIP1*/*CDKN1A*, *BAX*, *MDM2*,[15] and *PIG3* (G Bougeard, T Frebourg, unpublished data) genes.

We therefore analysed in this study the *TP53*, *hCHK2*, and *TP63* genes in 17 LFS/LFL families referred to our laboratory. These 17 families (table 1, figs 1 and 2) included four families fulfilling the complete criteria for LFS, five the criteria for LFL, as defined by Birch *et al*[3] and were therefore designated LFLb, and eight the criteria for LFL proposed by Eeles[4] and were therefore designated LFLe. For 15 index cases, we performed the *TP53* functional assay developed in yeast on cDNA derived from lymphocytes.[16 17] Briefly, the reporter yeast strain yIG397-RGC was co-transformed with PCR amplified *TP53* cDNA (between codons 53 and 364) and the gapped expression vector pSS16 linearised between codons 67 and 346, and cDNA was cloned in vivo by homologous recombination. The activation by wild type *TP53* of the reporter system, containing the ADE2 open reading frame and the *TP53* binding site RGC, changes the colour of the yeast colonies (red→white) and samples containing wild type *TP53* commonly give a background of 5-8% red colonies owing to PCR induced errors and the presence of an alternatively spliced *TP53* mRNA.[16 17] In 10 families, the percentage of red colonies (above 10%) suggested the presence of a heterozygous mutation, which was confirmed by sequence analysis of cDNA and/or genomic DNA (table 1, fig 1). Sequencing of exons 2 to 11 confirmed the absence of mutation in the five families with a normal *TP53* functional assay and, in one of the two families for which no RNA was available, allowed the detection of an additional mutation.

In the six families without detectable *TP53* germline mutation (fig 2), we then analysed the *TP63* gene. To screen for inactivating *TP63* mutations, we performed a functional assay in yeast. Transformation of the pCI51 plasmid[18] containing the wild type coding region corresponding to the isoform γ of TP63 into the yIG397-RGC yeast reporter strain resulted in white colonies. Transformation of two mutant pCI51 plasmids (with the mutation Leu264Ser or Cys269Ser generated by PCR induced mutagenesis), used as controls, resulted only in red colonies. In four index cases, for whom mRNA was available, the *TP63* cDNA was PCR amplified from lymphocytes between codons 18 and 434, cloned by homologous recombination into the gapped expression vector pCI51 linearised between codons 30 and 420, and transformed into yIG397-RGC; the percentage of red colonies (table 1) suggested the absence of heterozygous inactivating mutations. We also sequenced in the six families without detectable *TP53* mutation exons 2 to 15 of the *TP63* gene from genomic DNA, using primers described by Hagiwara *et al*,[19] and detected no nucleotide change. We then analysed the open reading frame of *hCHK2* by RT-PCR in the four index cases for whom mRNA was available. As recently highlighted by Sodha *et al*,[8] exons 10 to 14 of *hCHK2* have homologous fragments on numerous chromosomes, which limits analysis from genomic DNA, and the mutation screening should therefore be performed by RT-PCR. The entire coding region of *hCHK2* (1662 bp) was PCR amplified from cDNA derived from lymphocytes using primers (5'-TGT CTC GGG AGT CGG ATG TTG AGG CTC AGC-3') and (5'-GGA CAT TTC TTT CGT GTT CAA ACC ACG GAG-3'), and the PCR products were then submitted to a second stage PCR amplification generating three overlapping fragments which were sequenced. In all subjects, RT-PCR showed an in frame deletion of 87 bp, which was also observed in controls. Alignment, using the BLAST program (National Center for Biotechnology Information), of the *hCHK2* cDNA (accession number AF086904) to the genomic clones RP11-44G7 (accession number AL117330) and RP11-436C9 (accession number AL121825) derived from chromosome 22 showed that the deleted fragment corresponds to an exon (probably exon 9), indicating the presence of an alternative splicing of *hCHK2* in lymphocytes. Sequence analysis detected no germline *hCHK2* mutation in the four probands.

Table 1 Functional and sequencing analysis of the TP53, TP63, and hCHK2 genes in 17 LFS or LFL families

		TP53			*TP63*		*hCHK2*
Family	*Presentation*	*FASAY % of red colonies*	*Nucleotide change*	*Amino acid change*	*FASAY % of red colonies*	*DNA sequence*	*cDNA sequence*
F1	LFS	74	TGC → TAC	Cys141Tyr*	/	/	/
F2	LFS	17	CGC → GGC	Arg175Gly*	/	/	/
F3	LFS	12	1 bp (T) Del	212 FS Del†	/	/	/
F4	LFS	48	GGC → AGC	Gly245Ser‡	/	/	/
F5	LFLb	17	Intron 6 SD ag → aa§	225 FS Ins¶	/	/	/
F6	LFLb	ND**	ATG → ATA	Met237Ile*	/	/	/
F7	LFLb	53	CGT → TGT	Arg273Cys*‡	/	/	/
F8	LFLb	49	CCT → TCT	Pro278Ser*	/	/	/
F9	LFLb	6	WT	/	ND	WT	ND
F10	LFLe	22	CGC → CAC	Arg175His*‡	/	/	/
F11	LFLe	50	CGG → TGG	Arg282Trp*‡	/	/	
F12	LFLe	14	CGA → TGA	Arg306Stop‡	/	/	/
F13	LFLe	6	WT	/	8	WT	WT
F14	LFLe	4	WT	/	4	WT	WT
F15	LFLe	3	WT	/	4	WT	WT
F16	LFLe	2	WT	/	5	WT	WT
F17	LFLe	ND	WT	/	ND	WT	ND

*This missense mutation was shown to alter the transactivation of p21 and bax reporter constructs in yeast.[24]
†Frameshift deletion.
‡Germline mutation previously listed in data base from Christophe Beroud, Karim Debouche, and Thierry Soussi (http://perso.curie.fr/Thierry.Soussi/).
§Analysis of the cDNA showed that this mutation of the splicing donor site resulted in a partial retention of intron 6 owing to the activation of a cryptic site.
¶Frameshift insertion.
**Not done.
WT=wild type.

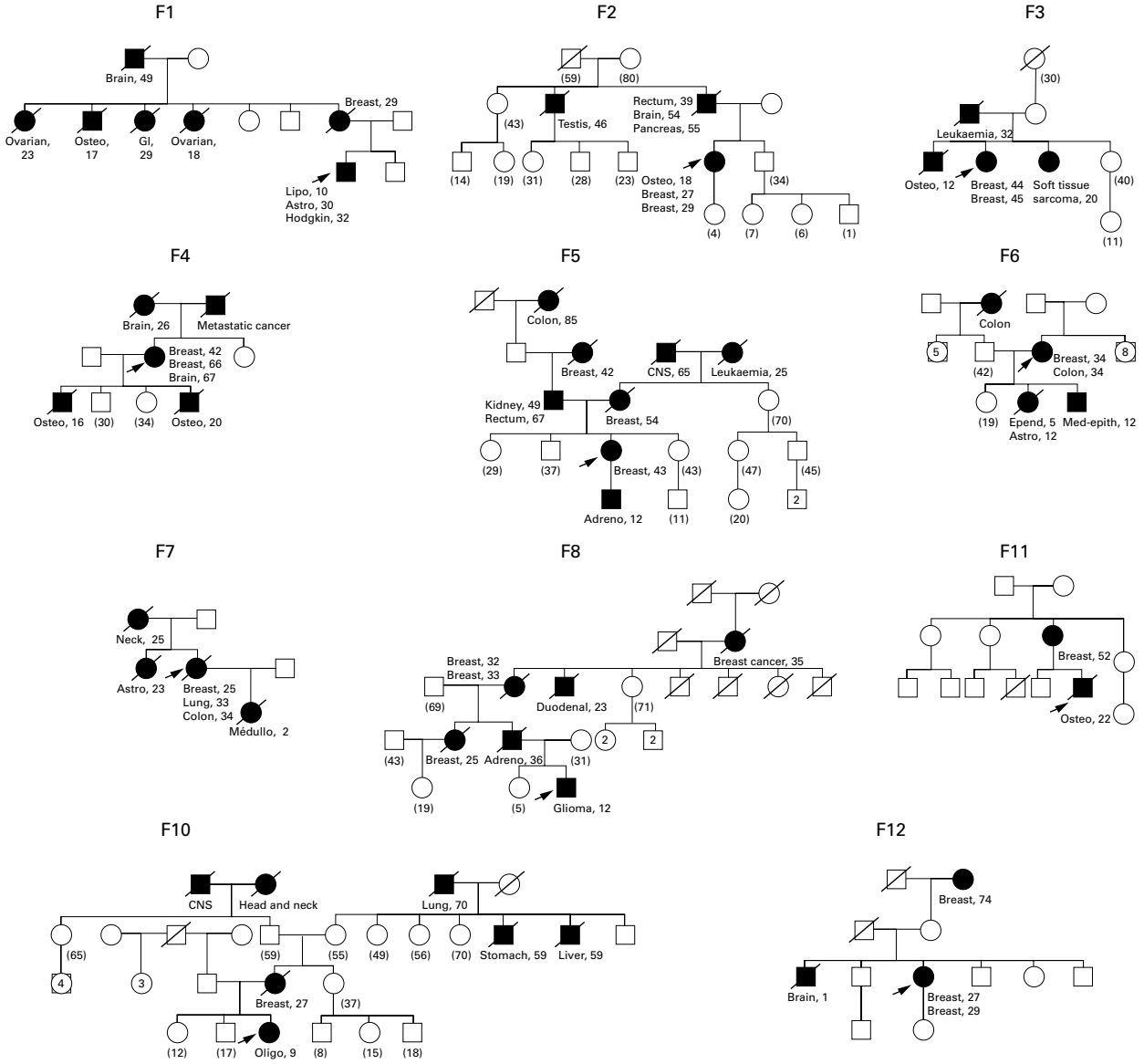

Figure 1 Partial pedigrees of 11 LFS/LFL families with a germline TP53 mutation. Filled symbols=affected subjects, open symbols=asymptomatic subjects. For each affected subject, the tumour (or site of the tumour) and age of diagnosis are indicated. The age of unaffected subjects is indicated in brackets. Adeno: adenocarcinoma of unknown origin; adreno, adrenocortical carcinoma; astro, astrocytoma; epen, ependymoma; GI, gastrointestinal tumour; histio, histiofibrosarcoma; lipo, liposarcoma; med-epith, medullo-epithelioma; medullo, medulloblastoma; oligo, oligodendroglioma; osteo, osteosarcoma. Germline TP53 mutations are shown in table 1.

We therefore identified in this study 11 germline *TP53* mutations, including six previously unreported germline mutations (table 1), in 4/4 LFS, 4/5LFLb, and 3/8 LFLe families (fig 1), confirming that the use of relaxed criteria decreases the probability of identifying a germline mutation. This study shows that the functional assay is an efficient method to detect germline *TP53* mutations since, in this series, it detected all the germline mutations. The recent identification in LFS families of germline mutations of *hCHK2*,[7] encoding a kinase whose substrates include Cdc25C[20] and TP53[9–11] strongly suggests that the critical defect in LFS is the constitutional alteration of the G1 and/or G2 checkpoints in response to DNA damage. In four LFL families, with no detectable germline *TP53* mutation, we did not detect any germline mutation of *hCHK2*. In this study, we also explored the involvement of *TP63*, one of the *TP53* homologues identified over the last two years. Somatic *TP63* mutations appear very rare in tumours.[18 19] Nevertheless, when we performed a functional assay in yeast on a *TP63* cDNA submitted to PCR induced mutagenesis, as previously described,[21] we were able to estimate the number of sites in which mutations could be detected as 528, indicating that *TP63* exhibited in vitro the same intrinsic sensitivity to mutations as *TP53*. Our study provides no evidence for the involvement of *TP63* in LFS. In contrast, germline *TP63* mutations were recently identified in the EEC (ectrodactyly, ectodermal dysplasia, and cleft lip) syndrome.[22] It could be argued that, in this study, the six families with no detectable mutation correspond either to other inherited forms of cancers or to the aggregation of sporadic cancers. Nevertheless, the association of early breast cancer with sarcoma or a CNS tumour in first degree relatives, observed in families F13, F14, and F15 (fig 2), is strongly suggestive of LFS. Therefore, our results indicate the involvement of other genes in LFS. If the medical benefit of presymptomatic testing in these families is not obvious considering the wide spectrum of tumours, in contrast identification of the germline alteration in affected subjects confirms on a molecular basis the diagnosis of LFS, which may have important

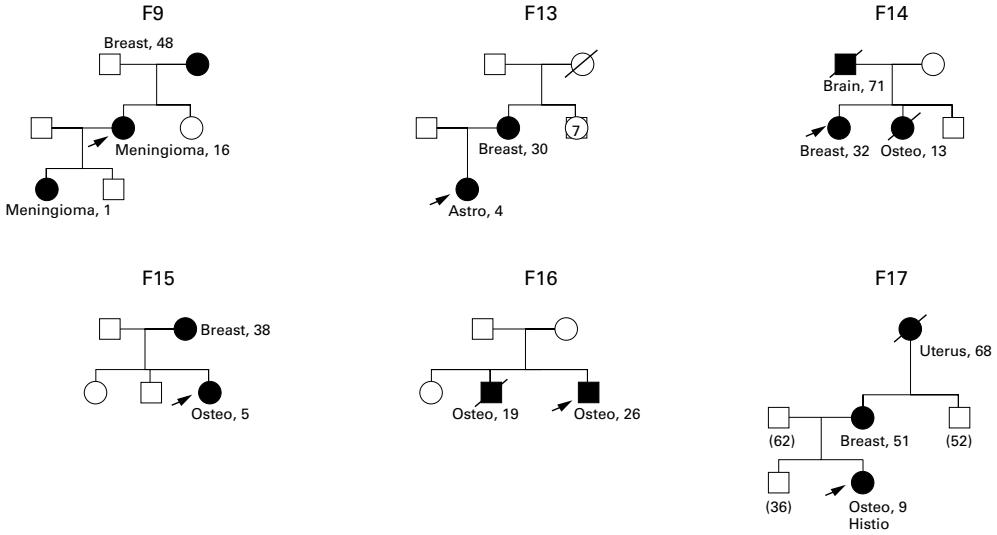

Figure 2 Partial pedigrees of six LFL families with no detectable germline TP53 mutation.

clinical implications. These patients, who have a high risk of developing multiple primary cancers,[23] may benefit from a regular clinical review. Furthermore, several studies have reported, in mutation carriers, the development of second tumours in the radiotherapy fields, which raises the question of the use of ionising radiation in these patients.[23] Therefore, the complete characterisation of the molecular basis of LFS will be important for the correct clinical management of these families.

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A distinct splice form of *APC* is highly expressed in neurones but not commonly mutated in neuroepithelial tumours

EDITOR—The *APC* tumour suppressor gene contains at least 21 exons, including four exons upstream of exon 1.[1 2] Alternative splicing involves at least seven exons, including the first five exons, and creates distinct splice forms of *APC* RNA.[1–4] Exon 1 contains an in frame stop codon upstream of its initiating methionine; hence only splice forms of *APC* that lack exon 1 allow exons 5' of exon 1 to be translated. Interestingly, splice forms lacking exon 1 are enriched in terminally differentiated tissues including brain,[5] intimating that protein isoforms containing domains encoded by the exons 5' to exon 1 may be important in cellular differentiation. This study evaluates neurones and glia of the rodent nervous system and asks which cell types express *APC* isoforms lacking exon 1.

Medulloblastomas and glioblastomas are neuroepithelial tumours derived from neuronal progenitor cells and glial cells, respectively.[6 7] Both tumour types occur at increased frequency in a subset of adenomatous polyposis coli (APC) patients[8]; APC patients developing these tumour types have a variant of APC known as Turbot's syndrome.[9] APC is an autosomal dominant disorder caused by the inactivation of one copy of the *APC* gene.[10 11] APC patients develop hundreds to thousands of adenomatous polyps and if the colon is not removed, colon carcinoma develops.[8] The relative risk of brain tumour formation is 23 times greater for APC patients when compared to the general population between the ages of 0 and 29.[12] This study also examines the genetic basis of neuroepithelial tumour formation and examines *APC* as a mutational target in medulloblastomas and glioblastomas. We assayed 41 sporadic glioblastomas and medulloblastomas, five cell lines derived from neuroepithelial tumour types, and one medulloblastoma from an APC patient to examine the possibility that mutations in the 5' exons of the *APC* gene are associated with neuroepithelial tumour formation. The mutation cluster region in exon 15 of the *APC* gene was also examined.

Adult rat cerebellum and spinal cord were dissected and stored at −20°C. Embryonic rat spinal cord was dissected from embryonic day 16 (E16) rats and stored at −20°C. Rat embryonic neurones were purified from E16 dorsal root ganglia as described by Kim *et al*.[13] Rat Schwann cells were purified from postnatal day 1 (P1) rats as described by Brockes and Raff.[14] Rat astrocyte cells were derived from P1 rats as described by McCarthy and de Vellis.[15]

Total RNA was extracted from samples by the guanidinium thiocyanate method.[16] cDNA was synthesised using random hexamers and Stratascript reverse transcriptase (Stratagene, La Jolla, CA) according to the manufacturer's instructions. The resulting cDNA was used as template in PCR reactions with an upstream primer, UP-GGAGAGAGAATGGAGGTGCTGC, derived from exon 0.3 of mouse *Apc*, and the downstream primer, RP-CTCTCTTTCTCAAGTTCTTCTA, in exon 3 of mouse *Apc*. UP and RP represent the universal and reverse primer, respectively, of M13 and were used for sequencing analysis. The upstream primer anneals specifically with nucleotides 154 to 175 of exon 0.3, nucleotide number 1 marking the 5' end of mouse *Apc* exon 0.3 (GenBank Accession No U66412). The downstream primer anneals specifically with nucleotides 401 to 422 of *Apc* exon 3, nucleotide number 1 being the beginning of the initiating methionine codon of mouse *Apc*. RT-PCR products were electrophoresed through a 3% agarose gel, stained with ethidium bromide, and photographed. Intensities of RT-PCR product bands were analysed by Image Quant software (Molecular Dynamics, Sunnyvale, CA).

All cell lines were obtained from the ATCC in Rockville, MD. The human astrocytoma cell line SW1088 (ATCC HTB12) was cultured in Liebovitz's L-15 medium (Gibco BRL, Grand Island, NY) with 10% fetal bovine serum (Hyclone, Logan, UT). The human glioblastoma cell line T98G (ATCC CRL1690) and the human medulloblastoma cell line DAOY (ATCC HTB186) were cultured in Minimum Essential Medium (Gibco BRL, Grand Island, NY) supplemented with 10% fetal bovine serum, 1% non-essential amino acids (Gibco BRL, Grand Island, NY), and 1% sodium pyruvate (Gibco BRL, Grand Island, NY). The human medulloblastoma cell lines D341 (ATCC HTB187) and D283 (ATCC HTB185) were cultured in Minimum Essential Medium-alpha formulation (Sigma, St Louis, MO) supplemented with 10% fetal bovine serum.

All human tissues were obtained with Institutional Review Board (IRB) approvals from the University of Cincinnati College of Medicine and University Hospital, and Instituto Nazionale per la Ricerca sul Cancro in Genova, Italy. Fresh frozen specimens of 17 medulloblastomas were obtained from Italy and the United States, as described by Badiali *et al*.[17] Twenty three paraffin embedded glioblastoma samples and five corresponding normal samples were obtained through the Department of Pathology and Laboratory Medicine, University of Cincinnati College of Medicine. Genomic DNA was prepared from paraffin embedded samples as described in Wright and Manow.[18] A paraffin embedded sample from a medulloblastoma and accompanying normal tissue were obtained from an APC patient treated at The Ohio State University James Cancer Hospital and Research Institute.

Normal genomic DNA was a gift from the laboratory of Anil G Menon, Department of Molecular Genetics, Biochemistry and Microbiology, University of Cincinnati College of Medicine. These samples were obtained with IRB approval from patients followed by the Hypertensive Clinics of the University of Cincinnati and the Veterans Administration Hospital, as well as from the community at large. DNA was extracted from peripheral blood as published in Su *et al*[19] using Puregene DNA Isolation Kit (Gentra Systems Inc, Minneapolis, MN).

PCR-single strand conformational polymorphism (SSCP) analysis of *APC* nucleotide positions 1959 to 4904 (exon 15A to 15I, according to Groden *et al*[10]) was performed using two different running conditions as described in Varesco *et al*[20] and Groden *et al*.[21] Exons 1-14 and the remainder of exon 15 were not screened owing to limited tumour DNA amounts and the clustering of most sporadic *APC* mutations within a small section of exon 15.

PCR-SSCP analysis of exon 0.3 of the *APC* gene was performed using primer pairs UP-CGAGGGG TACGGGGCTAGG and RP-ATGGGGAGCG CCCTGGTCC, UP-ATCCGCTGGATGCGGACC and RP-GGCAGCACCTCCATTCTGTCT, and UP-CTGTATTGGTGCAGCCCGCCA and RP-AAGA CAGTGCGAGGGAAAACCA. PCR-SSCP analysis of exon BS was performed using primer pairs UP-GGG GAGTCTGCTGAGAAAAG and RP-GCCTTTCAAT GGGGTAGAGC, and UP-GCTCTACCCCATT GAAAGGC and RP-ACCACCACTCACGCTCTCGA. PCR-SSCP analysis of exons 0.1 and 0.2 was performed using primer pairs UP-AGATGGCGGAGGGCAAG